JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2009, p. 3862–3870 Vol. 47, No. 12
0095-1137/09/$12.00 doi:10.1128/JCM.02094-08

Molecular and Phenotypic Evaluation of *Lichtheimia corymbifera* (Formerly *Absidia corymbifera*) Complex Isolates Associated with Human Mucormycosis: Rehabilitation of *L. ramosa*[▽]

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Received 31 October 2008/Returned for modification 15 January 2009/Accepted 8 September 2009

Thirty-eight isolates (including 28 isolates from patients) morphologically identified as *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) were studied by sequence analysis (analysis of the internal transcribed spacer [ITS] region of the ribosomal DNA, the D1-D2 region of 28S, and a portion of the elongation factor 1α [EF-1α] gene). Phenotypic characteristics, including morphology, antifungal susceptibility, and carbohydrate assimilation, were also determined. Analysis of the three loci uncovered two well-delimited clades. The maximum sequence similarity values between isolates from both clades were 66, 95, and 93% for the ITS, 28S, and EF-1α loci, respectively, with differences in the lengths of the ITS sequences being detected (763 to 770 bp for isolates of clade 1 versus 841 to 865 bp for isolates of clade 2). Morphologically, the shapes and the sizes of the sporangiospores were significantly different among the isolates from both clades. On the basis of the molecular and morphological data, we considered isolates of clade 2 to belong to a different species named *Lichtheimia ramosa* because reference strains CBS 269.65 and CBS 270.65 (which initially belonged to *Absidia ramosa*) clustered within this clade. As neotype *A. corymbifera* strain CBS 429.75 belongs to clade 1, the name *L. corymbifera* was conserved for clade 1 isolates. Of note, the amphotericin B MICs were significantly lower for *L. ramosa* than for *L. corymbifera* ($P < 0.005$) but were always ≤0.5 μg/ml for both species. Among the isolates tested, the assimilation of melezitose was positive for 67% of the *L. ramosa* isolates and negative for all *L. corymbifera* isolates. In conclusion, this study reveals that two *Lichtheimia* species are commonly associated with mucormycosis in humans.

Mucormycosis is a life-threatening infection that occurs in immunocompromised patients, diabetic patients with ketoacidosis, and immunocompetent patients after trauma exposure to contaminated soil (7, 18). The filamentous fungi responsible for these infections belong to the *Mucorales* order. About 20 different species have been shown to be pathogenic for humans (4). According to a recent review (19), the species that were the most frequent encountered were *Rhizopus* spp., *Mucor* spp., and *Cunninghamella* spp., while *Apophysomyces elegans* and *Absidia* spp. accounted for 6% and 5% of the cases, respectively. The true frequency is, however, difficult to assess because surveys are rare and determination of the species of the *Zygomycetes* class by standard mycological methods remains difficult. Indeed, all the genera and species within the family *Mucoraceae* (the *Absidia*, *Rhizopus*, *Mucor*, *Rhizomucor*, and *Apophysomyces* genera) shared similar morphological characteristics (6). The precise identification to the species level often requires the specific expertise usually available only at reference laboratories. The availability of molecular tools for taxonomic and identification purposes has changed the picture. Sequencing of various DNA targets has facilitated the recognition of phylogenetic species within the *Zygomycetes* (27, 28) and provided tools for DNA bar coding of these fungi (22). A revision of the genus *Absidia* was recently performed on the basis of phylogenetic, physiological, and morphological characteristics (10). A new family (*Mycocladiaceae*) and the genus *Mycocladus* were proposed to accommodate the three species *Mycocladus corymbifer* (formerly *Absidia corymbifera*), *M. blakesleeana*, and *M. hyalospora*. More recently, it was suggested that additional nomenclatural changes were necessary, and the names *Lichtheimiaceae* and *Lichtheimia* were proposed for the family and the genus, respectively (11).

The intraspecific variability of *Lichtheimia corymbifera* (formerly *A. corymbifera*) has been poorly evaluated so far. After the analysis of a small number of clinical isolates, we recently reported that some of the isolates morphologically identified as *L. corymbifera* had divergent internal transcribe spacer (ITS) sequences (21). Subsequently, the use of molecular identification on a routine basis for all isolates of the *Zygomycetes* collected at the French National Reference Center for Mycoses and Antifungals allowed us to uncover intraspecific sequence variability among isolates morphologically identified to be *L. corymbifera*. To further characterize the atypical isolates, we used three different DNA targets,

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▽ Published ahead of print on 16 September 2009.

TABLE 1. Isolates used in this study[a]

Strain no.	Strain[b]	Origin		Sex	Age (yr)	Underlying disease or condition	Country	GenBank accession no.		
		Source	Specimen					ITS	28S	EF-1α
1	CNRMA/F01-97	Human	Bone	M	17	None, trauma	France	FJ719392	FJ719414	FJ719469
2	CNRMA/F02-8	Human	Bronchial	F	46	Tx, lung	France	FJ719370	FJ719411	FJ719446
3	CNRMA/F02-33	Human	Skin	M	68	None, trauma	France	FJ719371	FJ719416	FJ719447
4	CNRMA/F02-62	Human	BAL fluid	M	23	HM	France	FJ719372	FJ719418	FJ719477
5	CNRMA/F03-62	Human	Lung biopsy	M	36	HM	France	FJ719373	FJ719425	FJ719479
6	CNRMA/F04-14	Human	Sputum	M	71	Cancer	France	FJ719375	FJ719409	FJ719449
7	CNRMA/F04-27	Human	Lung biopsy	M	42	HM	France	FJ719376	FJ719408	FJ719450
8	CNRMA/F04-35	Human	Skin	F	48	Aplasia	France	FJ719377	FJ719410	FJ719451
9	CNRMA/F04-61	Human	Skin	F	69	HM	France	FJ719378	FJ719423	FJ719480
10	CNRMA/F04-93	Human	Skin	M	83	None, trauma	France	FJ719380	FJ719419	FJ719478
11	CNRMA/F05-24	Human	Skin	M	62	HM	France	FJ719381	FJ719420	FJ719453
12	CNRMA/F05-79	Human	Skin	F	13	Cancer	France	FJ719382	FJ719421	FJ719454
13	CNRMA/F05-100	Human	Skin	F	51	Amputation, surgery	France	FJ719383	FJ719422	FJ719455
14	CNRMA/F06-32	Human	Nose	M	54	HM	France	FJ719384	FJ719424	FJ719456
15	CNRMA/F07-40	Human	Skin	M	29	None, trauma	Qatar	FJ719385	FJ719427	FJ719457
16	CNRMA/F07-63	Human	Cornea	F	87	None, trauma	France	FJ719386	FJ719430	FJ719458
17	CNRMA/F07-69	Human	Bone	M	27	None, trauma	Qatar	FJ719387	FJ719433	FJ719459
18	CNRMA/F07-70	Human	Ear	M	68	None	France	FJ719388	FJ719436	FJ719460
19	CNRMA/F07-76	Human	Skin	F	25	HM	France	FJ719389	FJ719434	FJ719461
20	CNRMA/F07-80	Human	Urine	F	65	Alcoholism, GI surgery	France	FJ719390	FJ719435	FJ719462
21	CNRMA/F07-88	Human	Skin	F	16	HM	France	FJ719391	FJ719437	FJ719463
22	CNRMA/F08-4	Human	BAL fluid	M	37	Corticosteroid use	France	FJ719393	FJ719438	FJ719464
23	CNRMA/F08-24	Human	Bronchial	M	40	HM	France	FJ719394	FJ719439	FJ719465
24	CNRMA/F08-54	Human	Skin	F	68	Tx, kidney	France	FJ719404	FJ719440	FJ719466
25	CNRMA/F09-5	Human	Bronchial	F	50	None, trauma	France	FJ719395	FJ719441	FJ719467
26	CNRMA/F09-12	Human	Bronchial	M	54	HM	France	FJ719402	FJ719442	FJ719468
27	CNRMA/F09-20	Human	Bronchial	F	18	Tx, lung	France	FJ719403	FJ719443	FJ719476
28	CNRMA/F03-82	Animal	Lung, chicken	NA	NA	Unknown	France	FJ719374	FJ719417	FJ719448
29	CBS 101040	Human	Cornea	Unknown	Unknown	Unknown	France	FJ719379	FJ719426	FJ719452
30	UMIP 1129.75	Env	Outdoor air	NA	NA	NA	Morocco	FJ719399	FJ719413	FJ719473
31	UMIP 1279.81	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	FJ719400	FJ719412	FJ719481
32	UMIP 1280.81	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	FJ719401	FJ719415	FJ719474
33	CBS 100.31	Animal	Aborted cow	NA	NA	NA	NA	FJ719398	FJ719429	FJ719472
34	CBS 269.65	Env	Hay	NA	NA	NA	NA	FJ719405	FJ719432	FJ719475
35	CBS 270.65	Unknown	Unknown	NA	NA	NA	NA	FJ719406	FJ719445	FJ719482
36	CBS 429.75	Env	Soil	NA	NA	NA	NA	FJ719407	FJ719444	FJ719483
37	BES227	Env	Hay	NA	NA	NA	France	FJ719397	FJ719428	FJ719470
38	BES228	Env	Hay	NA	NA	NA	France	FJ719396	FJ719431	FJ719471

[a] Culture collection abbreviations: CNRMA/F, Centre National de Référence Mycologie et Antifongiques-Filamentous Fungi Collection, Institut Pasteur, Paris, France; UMIP, Pasteur Institut Collection of Fungi, Paris, France; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IHEM, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium. Other abbreviations: M, male; F, female; Tx, transplantation; Env, environment; NA, not applicable; BAL, bronchoalveolar lavage; HM, hematological malignancy; GI, gastrointestinal.

[b] Other collection numbers: CNRMA/F01-97 = CBS 120805; CNRMA/F02-62 = CBS 120580; CBS 100.31 = IHEM 3809 = NRRL 2982; UMIP 1129.75 = IHEM 10339; CBS 101040 = UMIP 2018.91; CBS 269.65 = ATCC 11613 = NRRL 1332; CBS 429.75 = ATCC 46771 = NRRL 2981.

which allowed us to confirm that *L. corymbifera* is a species complex.

MATERIALS AND METHODS

Isolates. The 38 isolates used in this study are presented in Table 1. Most of the isolates were of clinical origin ($n = 28$) and were mostly from immunocompromised patients ($n = 16$), but they were also from immunocompetent patients who became infected after injury ($n = 7$) or surgery ($n = 2$) and for whom the clinical presentations were skin lesions (with or without osteitis; $n = 12$) or pulmonary ($n = 6$), rhinocerebral ($n = 2$), and disseminated ($n = 5$) infections. The pathogenic role of the fungus in the three remaining cases was uncertain. In addition, one isolate was recovered from the lung of a chicken suspected of having pulmonary aspergillosis, and two isolates were cultured from hay in the region of Besançon, France. All isolates have been identified as *L. corymbifera* on the basis of morphological findings (white to greyish expanding colonies, branched mycelium, and the presence of stolons and rhizoids) and microscopy (spherical to pyriform sporangia, funnel-shaped apophyses, and smooth-walled endospores).

The remaining seven isolates were strains of *L. corymbifera* obtained from international culture collections (Centraalbureau voor Schimmelcultures [CBS] and the Collection of Fungi from the Pasteur Institute Collection). Additionally, two isolates belonging to other *Lichtheimia* species (*L. blakesleeana* CBS 100.28 and *L. hyalospora* CBS 173.67) were used for DNA sequence analysis. All isolates were stored as spore suspensions at −20°C in 40% glycerol. All isolates were subcultured for 3 to 6 days on 2% malt extract agar (MEA) at 30°C for macroscopic and microscopic examination.

Molecular study. (i) Extraction, amplification, and sequencing. Mycelium was grown in 20 ml of RPMI 1640 medium with L-glutamine but without sodium bicarbonate (Sigma-Aldrich, Saint Quentin Fallavier, France) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma-Aldrich). After 48 h of continuous agitation (100 rpm) at 30°C, the mycelium was recovered, washed twice with a 0.9% NaCl solution, and stored at −20°C until extraction.

Genomic DNA extraction was performed as described previously (22) with approximately 200 mg of mycelium, and the DNA was stored at −20°C. The complete ITS1-5.8S-ITS2 region of the ribosomal DNA (rDNA) was amplified with primer pair V9D (5′-TTAAGTCCCTGCCCTTTGTA-3′) and LS266 (5′-GCATTCCCAAACAACTCGACTC-3′) (9). The D1-D2 region of the large-subunit rDNA was amplified with primer pair NL-1 (5′-GCATATCAATAAG CGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (14). A small region of the 5′ elongation factor 1α (EF-1α) nuclear gene was amplified with primers MEF-11 (5′-AAGAAGATTGGTTTCAACCC-3′) and MEF-41 (5′-GCACCGATTTGACCAGGRTGG-3′) (17).

The PCR amplification of ITS and 28S was done as described previously (22) in an iCycler thermocycler (Bio-Rad, Hercules, CA). For the amplification with primers MEF-11 and MEF-41, the PCR mixture (50 μl) contained 3 μl of the extracted genomic DNA, 1× PCR buffer (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM $MgCl_2$, 0.25 μM of each primer, 0.25 mM of each deoxynucleoside triphosphate (Roche), and 1.25 U of AmpliTaq DNA polymerase (Roche). The PCR conditions were predenaturation at 94°C for 5 min; 40 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final incubation at 72°C for 7 min. The PCR products were then sequenced at the Institut Pasteur sequencing facility by using a BigDye Terminator (version 1.1) kit (Applied Biosystems, Foster City, CA) and each of the primer pairs used for amplification on an ABI Prism 3730 XL DNA analyzer (Applied Biosystems).

(ii) Sequence analysis. A consensus sequence was computed from the forward and reverse sequences by using the ChromasPro program (version 1.33; Technelysium, Helensvale, Queensland, Australia), and multiple-sequence alignments were performed with the Clustal W program (26). The analysis treated gaps (indels) as a fifth state character. To determine the percentage of identical residues between each pair of sequences, identity matrices for each set of data were generated with BioEdit software (Isis Therapeutics, Carlsbad, CA). The percent similarity represents the number of identical sites divided by the length of the longest sequence (sites at which a gap was present in both sequences were removed). Single-locus cladograms were constructed by the neighbor-joining method with the pairwise-deletion option (20) in the MEGA (version 3.1) computer program (13). A combined three-locus analysis was also performed. *Rhizomucor pusillus* (CNRMA/F09-7) was chosen as the outgroup, and the robustness of the branches was assessed by bootstrap analysis with 1,000 replicates.

Carbon source assimilation profiles. Carbon source assimilation profiles were determined with a commercial kit (ID32C system; bioMérieux, Marcy, l'Etoile, France), as described previously (23). Briefly, isolates were cultured for 7 days on Sabouraud agar slants at 30°C to obtain sufficient sporulation. The spores were transferred to API C medium (bioMérieux) to achieve a final concentration of 5×10^5 spores/ml, and 135 μl was distributed into each well. The results were read visually after 72 h of incubation at 30°C. Weak growth was considered positive. A functional analysis by use of an agglomerative clustering method (by use of the unweighted-pair group method with arithmetic mean algorithm) was performed with BioloMICS (Biological Manager for Identification, Classification and Statistics) software (version 7.2.5; BioAware, Hannut, Belgium) to group the isolates and the carbon assimilation results at the same time.

In vitro susceptibility testing. All isolates were subcultured on Sabouraud dextrose agar (supplemented with 0.02% chloramphenicol) prior to testing to ensure purity and viability. Pure powders of known potency of amphotericin B (Sigma-Aldrich), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), itraconazole (Janssen-Cilag, Issy-les-Moulineaux, France), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), flucytosine (Sigma-Aldrich), terbinafine (Novartis Pharma AG, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), and micafungin (Astellas Pharma, Osaka, Japan) were used. In vitro susceptibility was determined by a broth microdilution technique, according to the guidelines of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for the testing of conidium-forming molds (24), but with some modifications. Briefly, microplates containing the eight antifungal drugs were prepared in batches and stored frozen at −20°C. The final concentrations were 0.125 to 64 mg/liter for flucytosine and 0.015 to 8 mg/liter for all other drugs. Testing was performed in RPMI 1640 medium supplemented with 2% glucose for all drugs except amphotericin B, which was tested in AM3 medium, with a final inoculum size of 10^5 CFU/ml. MIC endpoints were determined on an automated microplate reader spectrophotometer (Multiscan RC-351; Labsystems Oy, Helsinki, Finland) after 24 h or 48 h of incubation (an optical density of >0.15 was required for the drug-free control wells) at 35°C. The MIC endpoint was defined as a reduction in growth of 80% or more compared to the amount of growth in the drug-free well for all drugs except amphotericin B, for which an endpoint of a 90% reduction was used. Two reference strains, *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, were included in each set of determinations to ensure quality control.

Morphological study. A detailed morphological study was performed with 11 isolates (5 isolates randomly chosen from each clade [see below] plus isolate CNRMA/F05-100). Isolates were cultured on MEA at 30°C, and the macroscopic morphology was described after 3 to 4 days of incubation. Microscopic examination was done with cultures grown for 5 to 9 days after they were mounted in water with 1% gelatin. The different structures (sporangia, columellae, and sporangiospores) were examined. The sporangiospores were measured with a DM LB2 optical microscope (Leica Microsystèmes SAS, Rueil-Malmaison, France) with interferential contrast and a Leica D5000 microscope coupled with Leica Application Suite software, which comprises the Multifocus and the Interactive Measurement modules (precision, 0.01 μm). For each isolate, approximately 100 sporangiospores were measured, and the ratio between the length and the width was calculated.

Statistical analysis. The distributions of the MICs were compared by a nonparametric test (Mann-Whitney). The mean spore length, width, and length/width ratio were calculated for the *L. corymbifera* isolates ($n = 613$) and the *L. ramosa* isolates ($n = 601$) and were compared by an unpaired *t* test. Analyses were performed with Prism (version 3.00) software for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a *P* value of ≤0.05.

RESULTS

Molecular data. The sequences of the whole ITS1-5.8S-ITS2 region, the D1-D2 domain of 28S, and a partial region of the EF-1α gene were determined for the 38 isolates (total length, approximately 68,000 bp). For the ITS locus, the sequences (starting at the ITS1 primer position and ending at the ITS4 primer position) ranged from 741 to 865 nucleotides in length: 613 to 617 nucleotides for the 28S D1-D2 domain and 439 nucleotides for the EF-1α locus. Two well-delimited clades were obtained with all of the single-locus distance trees generated (Fig. 1 to 3) and with the tree obtained when the three loci were combined (Fig. 4). As all individual isolates were grouped together in one clade for each locus, it was possible to consider the two clades two different species, in accordance with the principles of the genealogical concordance of phylogenetic species recognition (25).

Analysis of the ITS data matrix revealed a high degree of nucleotide sequence similarity (more than 98%) within clade 1, with the exception of that for isolate CNRMA/F05-100, which showed nucleotide sequence differences of more than 20% with the sequences of the other clade 1 isolates. Within clade 2, the maximum difference was observed between the subgroup consisting of isolates CNRMA/F02-8 and CNRMA/F04-35 and the rest of the clade 2 isolates (91.5 to 93.5% similarity). The sizes of the ITS sequences differed between the two clades (763 to 770 bp and 841 to 865 bp for clade 1 and clade 2, respectively). Greater than 99% similarity within the clade 1 sequences and only small variations (∼2%) within the clade 2 sequences were observed when the 28S domain sequences were analyzed, and differences of less than 2% were observed within each clade when the EF-1α locus was analyzed (Table 2). The highest degree of sequence variability between clade 1 and clade 2 was observed for the ITS locus (34%, 5%, and 7%

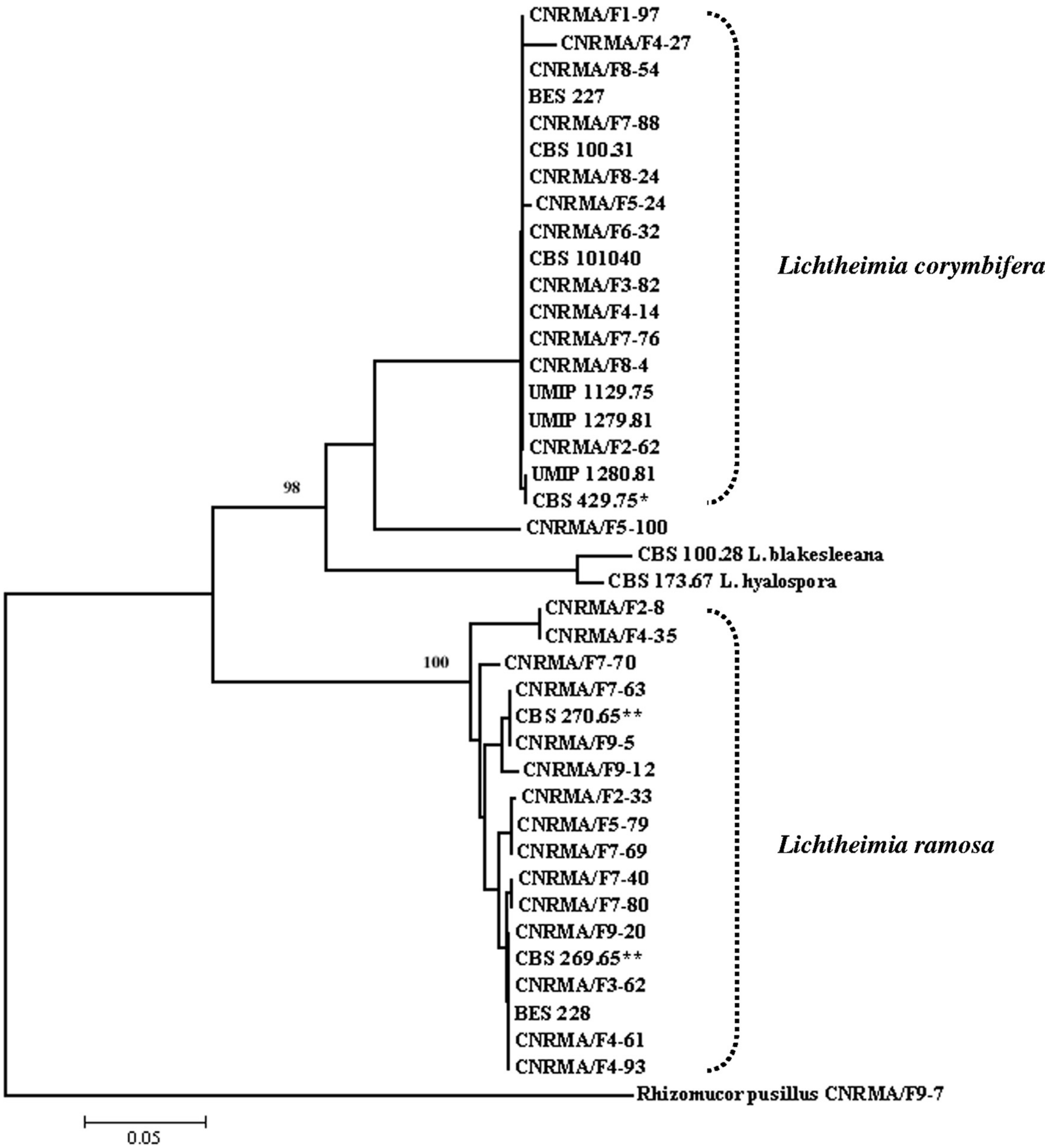

FIG. 1. Neighbor-joining analysis based on the complete sequences of ITS1-5.8S-ITS2. Bootstrap values from 1,000 replicates are indicated at the nodes separating the two clades. *Rhizomucor pusillus* was used as the outgroup. The bar indicates the number of substitutions per site. *, neotype strain of *Absidia corymbifera*; **, reference strains of *Absidia ramosa*.

variability for the ITS, 28S, and EF-1α gene regions, respectively).

Clade 1 corresponded to *Lichtheimia corymbifera* because it included neotype strain *Absidia corymbifera* CBS 429.75. Clade 2 isolates were designated *L. ramosa* because reference isolates CBS 269.65 and CBS 270.65 (which initially belonged to the species *Absidia ramosa*) (8) clustered within this clade.

Comparative morphology of *L. corymbifera* and *L. ramosa* isolates. After 3 to 4 days of incubation on MEA, colonies of all isolates were expanding but differences in the growth patterns were observed. The *L. corymbifera* isolates exhibited compact growth, while the *L. ramosa* isolates had a more effuse mycelium. No significant differences in the morphologies of the sporangia and columellae or the branching patterns of the sporangiophores were observed between the two species (Fig. 5). The sporangiospores of the *L. corymbifera* isolates were smooth and hyaline, whereas those of the *L. ramosa* isolates were smooth but slightly colored. More importantly, the sporangiospores of the *L. corymbifera* isolates were ellipsoid (2.73 by 2.24 μm), while those of the *L. ramosa* isolates were long ellipsoid (3.06 by 2.18 μm) (Fig. 5), with significant ($P < 0.0001$) differences in terms of length, width, and the length/width ratio (1.41 versus 1.22, respectively) being observed.

Comparison of other phenotypic characteristics between the two species. Melezitose and palatinose were assimilated by 67% and 33% of the *L. ramosa* isolates, respectively, while

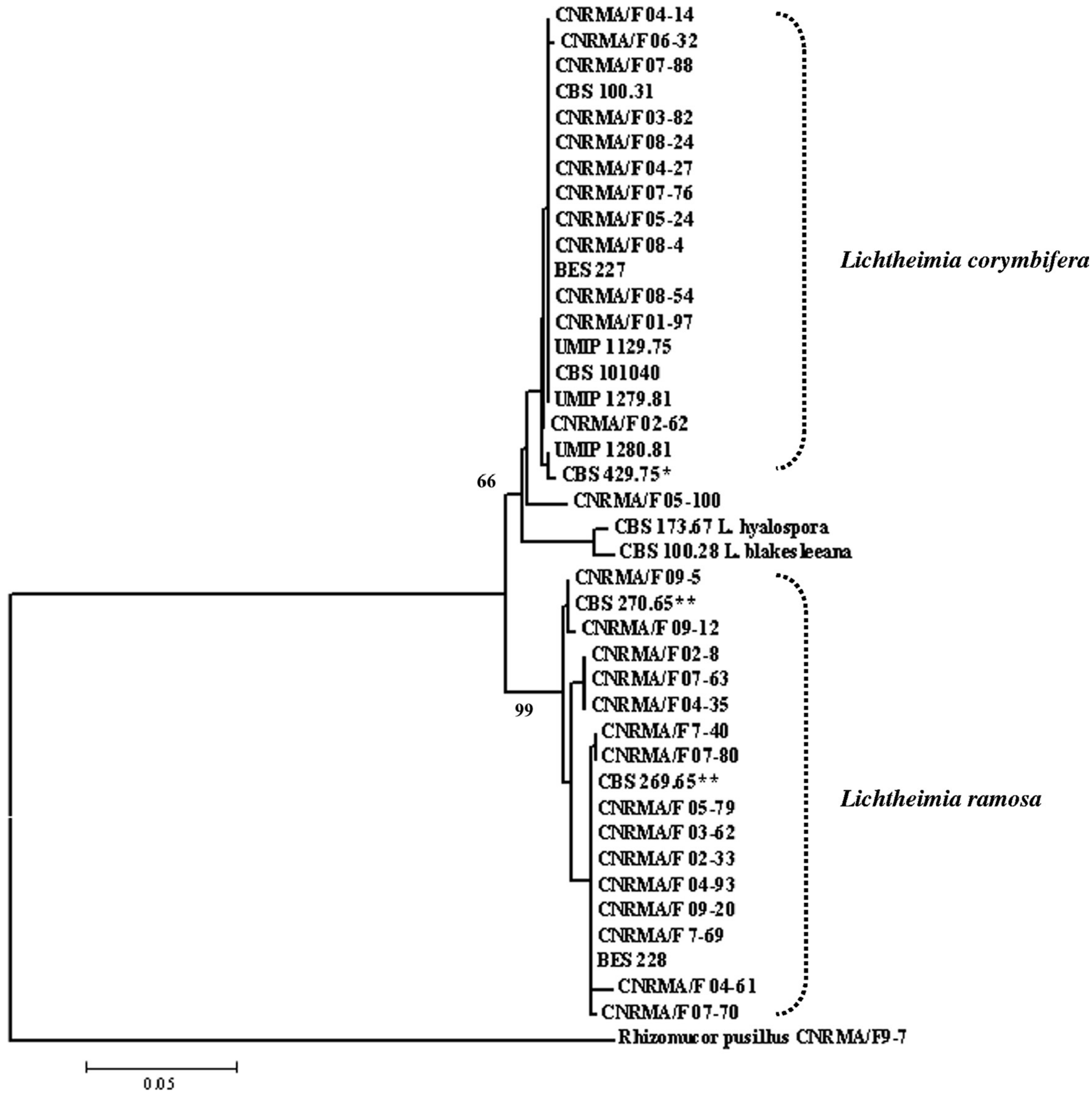

FIG. 2. Neighbor-joining analysis based on partial 28S sequences. Bootstrap values from 1,000 replicates are indicated at the nodes separating the two clades. *Rhizomucor pusillus* was used as the outgroup. The bar indicates the number of substitutions per site. *, neotype strain of *Absidia corymbifera*; ** reference strains of *Absidia ramosa*.

none of the *L. corymbifera* isolates tested assimilated those two carbon sources (Table 3). There were no additional differences in carbon source assimilation profiles that could discriminate between the two species.

The susceptibilities of the clinical isolates to eight antifungal drugs were determined. All isolates exhibited high flucytosine MICs (>64 μg/ml), caspofungin MICs (>8 μg/ml), and micafungin MICs (>8 μg/ml); and all but one isolate had a high voriconazole MIC (>8 μg/ml). Differences in the itraconazole MICs (range, 0.25 to 16 μg/ml), posaconazole MICs (range, 0.125 to 4 μg/ml), and terbinafine MICs (range, 0.125 to 2 μg/ml) were observed among the isolates; but there were no significant differences by species. A significant difference in the amphotericin B MIC distribution was observed between the two species (0.125 to 0.5 μg/ml for the *L. corymbifera* isolates versus 0.03 to 0.25 μg/ml for the *L. ramosa* isolates; $P < 0.005$). It should be noted, however, that the MIC_{50} differed by only 2 $\log_2$ dilutions.

Finally, there was no difference between the two species in terms of the underlying diseases of the patients from whom they were recovered (hematological malignancies, solid cancer, organ transplantation, or a lack of immunosuppression) or the clinical presentations that they caused (cutaneous, pulmonary, and disseminated infections).

The main molecular and phenotypic characteristics that differentiated the *L. ramosa* isolates from the *L. corymbifera* isolates are presented in Table 3.

DISCUSSION

Recently, a revision of the genus *Absidia* on the basis of the phylogenetic, physiological, and morphological characteristics

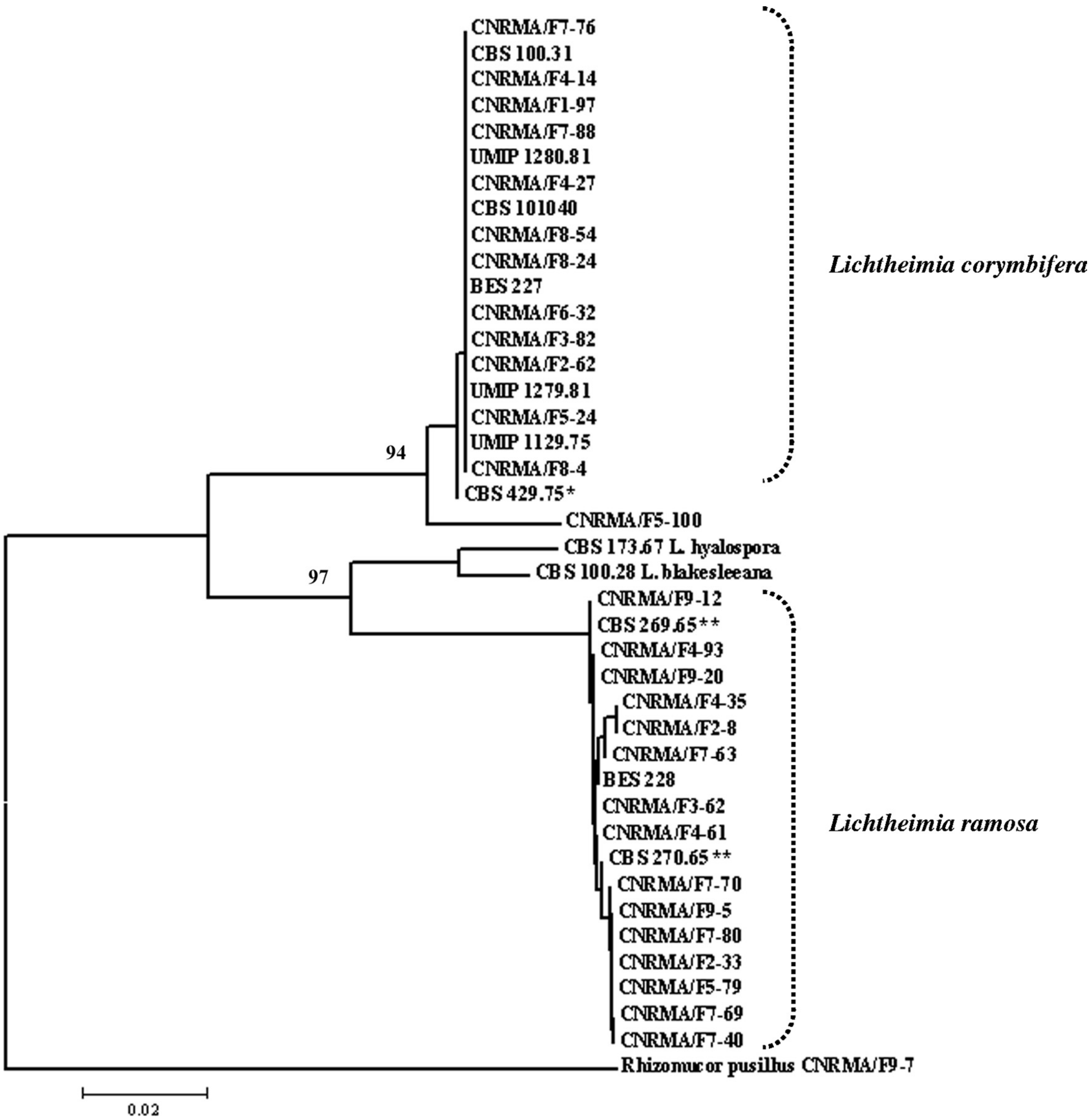

FIG. 3. Neighbor-joining analysis based on partial EF-1α sequences. Bootstrap values from 1,000 replicates are indicated at the nodes separating the two clades. *Rhizomucor pusillus* was used as the outgroup. The bar indicates the number of substitutions per site. *, neotype strain of *Absidia corymbifera*; **, reference strains of *Absidia ramosa*.

of 16 species was conducted (10), and nomenclatural changes were proposed (11). The three thermotolerant *Absidia* species (*A. corymbifera*, *A. blakesleeana*, and *A. hyalospora*) are now classified in the genus *Lichtheimia*. *L. corymbifera* was the only species pathogenic for humans. Although *L. corymbifera* is reported to be responsible for only 5% of the human cases of zygomycosis (19), this figure should be considered with caution because of a lack of surveys and because identifications are mostly based on morphology (12). The use of molecular identification (2) will be important for an accurate assessment of the epidemiology.

Phylogenetic species recognition in the *Mucorales* order is performed by sequencing rDNA genes (18S, 28S, and ITS), as well as the actin and EF-1α genes (10, 17, 27, 28). For identification (DNA bar coding) of this group of fungi, ITS is a good molecular target (22). The recent guidelines published by the Clinical and Laboratory Standards Institute (3) recommend the use of ITS sequencing as a first-line method for the identification of species within the *Mucorales*, an approach that was further approved by another international consortium of experts (1). Our routine use of ITS sequencing for the molecular identification of filamentous fungi allowed us to notice that some isolates morphologically identified as *L. corymbifera* had divergent ITS sequences (21). To further characterize these isolates, two other loci (28S and EF-1α) were sequenced for all the isolates initially identified as *L. corymbifera*. On the basis of those data, the morphospecies *L. corymbifera* appeared to be a species complex that included at least two clades. Due to the low level of sequence similarity (maximums, 66, 95, and 93% for ITS, 28S, and EF-1α, respectively) between the two clades

FIG. 4. Neighbor-joining analysis based on the combined data set. Bootstrap values from 1,000 replicates are indicated at the nodes separating the two clades. *Rhizomucor pusillus* was used as the outgroup. The bar indicates the number of substitutions per site. *, neotype strain of *Absidia corymbifera*; **, reference strains of *Absidia ramosa*.

TABLE 2. Summary of intraspecific and interspecific DNA sequence variability of *L. corymbifera* and *L. ramosa* isolates for ITS1-5.8S-ITS2, 28S, and EF1-α[a]

DNA locus	% Similarity		
	Within *L. corymbifera*	Within *L. ramosa*	Between *L. corymbifera* and *L. ramosa*
ITS	98–100	91.5–100	65.2–66.3
28S	99.1–100	97.8–100	93.8–95.4
EF-1α	98.6–100	99.3–100	92.4–93.1

[a] Strain CNRMA/F05-100 was excluded from the analysis.

and because individual isolates clustered in the same clade for each of the three loci, clade 2 isolates represent a separate species within the *L. corymbifera* complex that we named *L. ramosa*. It is noteworthy that the sequences of these three loci are more diverse within *L. ramosa* than within *L. corymbifera*. This heterogeneity should be further confirmed by analysis of additional *L. ramosa* isolates. One isolate (CNRMA F05-100) had sequences divergent from the sequences of both *L. corymbifera* and *L. ramosa* and was thus not assigned to either of those two species.

To briefly summarize the complex nomenclatural history of the species *L. ramosa* (*A. ramosa*) (15), in 1886, Lindt described this species in the genus *Mucor* Micheli 1729. In 1890, Zopf placed the species in the genus *Rhizopus* Ehrenberg 1820.

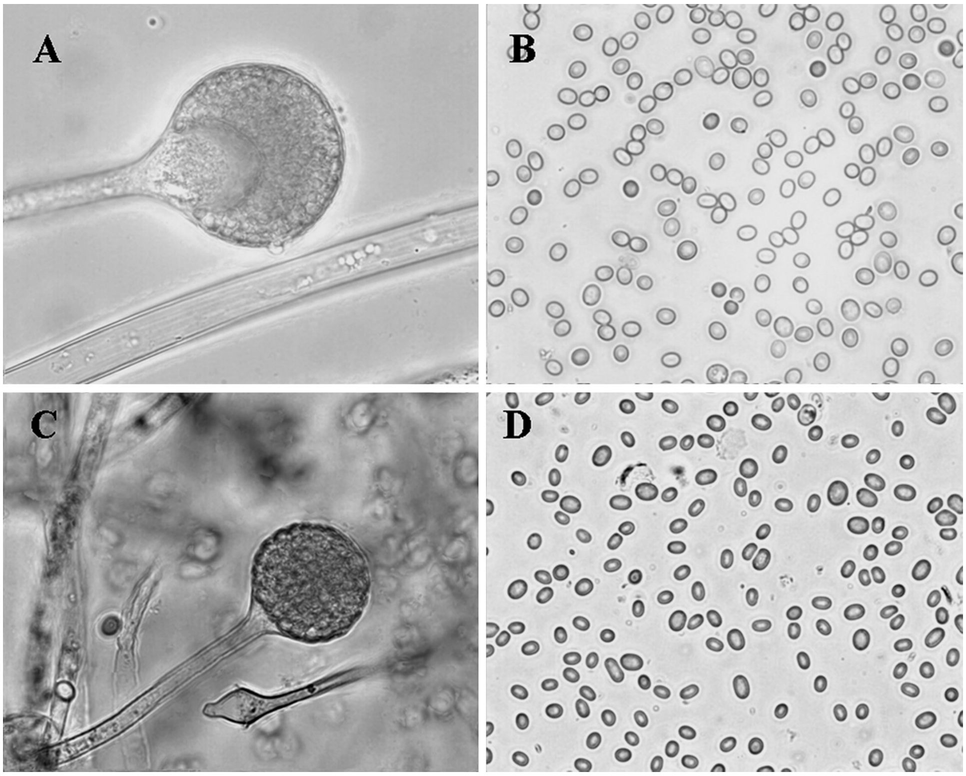

FIG. 5. Morphological characteristics of *L. corymbifera* isolates (CNRMA/F08-54) (A and B) and *L. ramosa* isolates (CNRMA/F05-79) (C and D). (A and C) Sporangia. Magnifications, ×400. (B and D) Sporangiospores. Magnifications, ×1,000.

In 1903, Vuillemin described the genus *Lichtheimia*, which comprised the type species *L. corymbifera* (29) and *L. ramosa*. In 1908, however, Lendner placed both species in the genus *Absidia* Van Thieghem 1876. Despite the morphological differences underlined by Ellis and Hesseltine (8), subsequent studies proved this distinction difficult and *A. ramosa* was reduced to being synonymous with *A. corymbifera* (16).

Both species infect humans and cannot be differentiated in terms of the hosts that they infect or the types of disease that they cause. *L. corymbifera* and *L. ramosa* are very similar both macroscopically and microscopically, but some differences that delineate the two species were uncovered. First, by culturing isolates on MEA plates at 30°C for 3 to 4 days, compact growth characterizes *L. corymbifera*, while more effuse growth is suggestive of *L. ramosa*. The sporangiospores of *L. corymbifera* are smooth, hyaline, and ellipsoidal when they are mature, while those of *L. ramosa* are smooth, lightly colored, and more ellipsoidal, a finding consistent with the earlier description by Ellis and Hesseltine (8). Carbohydrate assimilation can be used for *Zygomycetes* identification (23) but was not useful for the differentiation of *L. corymbifera* from *L. ramosa*. Indeed, only if palatinose or melezitose assimilation were positive could we suspect the species to be *L. ramosa*. Likewise, the antifungal susceptibility profiles were undistinguishable between the two *Lichtheimia* species, whereas they can be used to distinguish some species within other genera (5).

TABLE 3. Main molecular, morphological, and physiological characteristics of *L. corymbifera* and *L. ramosa* isolates[a]

Characteristic	*L. corymbifera*	*L. ramosa*
ITS sequence length (bp)[b]	763–770	841–865
Growth	Compact	Effuse
Sporangiospores	Ellipsoid	Long ellipsoid
Assimilation of melezitose (% of isolates)	0	67
% of isolates for which amphotericin B MIC is ≤0.125 μg/ml	20	87

[a] The numbers of isolates used for determination of these characteristics were 10 for growth and sporangiospore size, 37 for ITS sequence length and assimilation profile, and 26 for antifungal susceptibility testing. Strain CNRMA/F05-100 was excluded from the analysis.
[b] The number of base pairs of the region located between primers ITS1 and ITS4.

The results of the present study clearly show that molecular, biological, and morphological characteristics support the separation of the two species, even if their detection by classical methods remains difficult. In conclusion, *L. ramosa* represents a species distinct from *L. corymbifera* and is thus another *Lichtheimia* species responsible for mucormycosis in humans.

ACKNOWLEDGMENTS

We are grateful to Monique Coutanson and Bernard Papierok from the Pasteur Institute Collection of Fungi for providing some of the reference strains. Other clinical isolates were studied as part as a nationwide survey of invasive mycosis in France. Members of the French Mycoses Study Group who sent isolates used in this study were as follows (in alphabetical order by city in France): C. Duhamel (Caen), D. Pons (Clermont-Ferrand), E. Forget (Clichy), F. Dalle (Dijon), B. Sendid (Lille), F. de Monbrison (Lyon), F. Gay-Andrieu (Nantes), M. Gari-Toussaint (Nice), C. Lacroix (Paris), C. Kauffmann-Lacroix (Poitiers), D. Toubas (Reims), P. Cahen (Suresnes), and F.

Benaoudia (Troyes). We are also grateful to Saad J. Taj-Aldeen (Doha, Qatar) for sharing some clinical isolates and to Gabriel Reboux for providing two environmental isolates. We thank Laure Diancourt and Coralie Tran from the Institut Pasteur sequencing program for technical help.

We thank the Institut Pasteur sequencing program for financial support (Genopole PF-8).

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